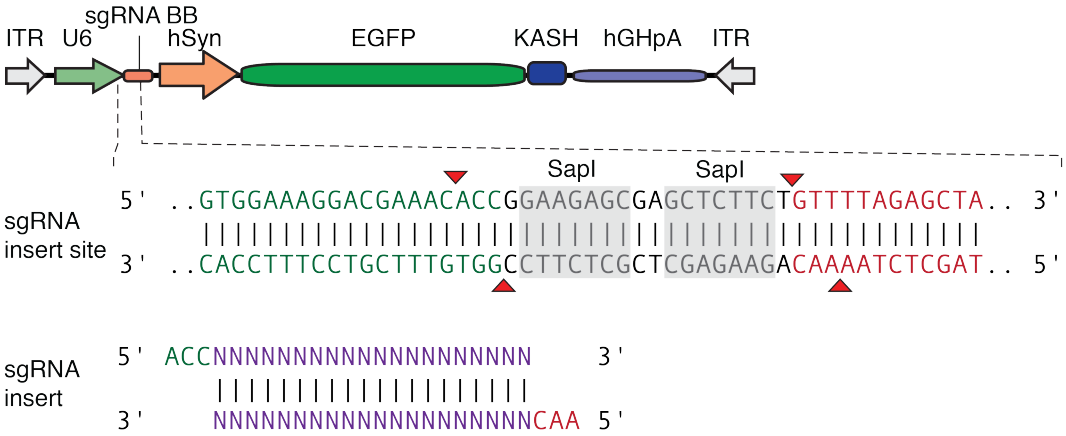
AAV:ITR-U6-sgRNA(backbone)-hSyn- EGFP-KASH-hGHpA-ITR

This plasmid facilitates fluorescence assisted sorting of cells and nuclei in addition to sgRNA expression. This plasmid contains two expression cassettes: EGFP-KASH and an sgRNA backbone for cloning new targeted plasmids. The plasmid can be digested with SapI creating sticky ends for ligation of annealed and phosphorylated DNA oligonucleotides designed based on the target site sequence (20bp).



Cloning protocol

Backbone vector digestion

Vector backbone DNA (1 µg)	x µl
FastDigest 10x buffer (Thermo)	5 µl
SapI (LguI) FastDigest (Thermo)	4 µl
FastAP (Thermo)	1 µl
H2O	x µl
Total	50 µl

-Incubate at 37°C for 1 hour.
-Purify the digested vector by gen purification (QIAquick Gel Extraction Kit (Qiagen)).

Oligo phosphorylation/annealing reaction

Top oligo (100 µM)	1 µl
Bottom oligo (100 µM)	1 µl
Buffer A 10x (Thermo)	2 µl
ATP (25 mM)	1 µl
H_2O	14 µl
PNK (Thermo)	1 µl
Total	20 µl

-Incubate at 37°C for 30 min, 95°C for 5 min, ramp to 4°C by 0.1°C/sec.

Ligation

Digested vector (25 ng)	x µl
Phosphorlyated/annealed olgios (1:50)	1 µl
Rapid Ligation Buffer 2x (Enzymatics)	1 µl
T7 Ligase (Enzymatics)	0.75 µl
H20	x µl
Total	10 µl

-Prepare a negative control reaction by excluding Digested vector.
-Incubate at room temperature for 10 min.

Transformation

Transform 1 µl of ligation reaction mixture in 25 µl of Z-competent (Zymo) Stbl3 E. coli (Life).

Sequencing

Prepare a mini prep for 1-4 colonies (QIAprep Spin Miniprep Kit (Qiagen)). Sequence purified plasmid using a U6-Forward sequencing primer: 5'-GAGGGCCTATTTCCCATGATTC-3'.

Reagents:

SapI (LguI) FastDigest (Thermo) # FD1934
FastAP (Thermo) # EF0654
PNK (Thermo) # EK0031
QIAquick Gel Extraction Kit (Qiagen) # 28704
T7 Ligase (Enzymatics) # L6020L
Z-competent (Zymo) # T3001
Stbl3 E. coli (Life) # C7373-03
QIAprep Spin Miniprep Kit (Qiagen) # 27104